Liver-Directed Gamma Interferon Gene Delivery in Chronic Hepatitis C

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Hepatitis C virus (HCV) is a member of the genus Flavivirus in the family Flaviviridae. HCV infection is a leading cause of chronic viral hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide and is the most common reason for liver transplantation in the United States (1). Although treatment for chronic hepatitis C has significantly been improved by the introduction of pegylated alpha interferon (IFN-α) and ribavirin combination therapy, it is associated with considerable side effects and up to 50% of HCV genotype 1-infected patients do not respond to therapy (8, 21). Therefore, the quest for improved or alternative treatment regimens continues.

Interestingly, recent studies using the chimpanzee model of HCV infection suggested that HCV induces IFN-α early after infection. HCV appears to be resistant to IFN-α’s antiviral effects, however, because it persists in the liver despite early induction of 2′5′ oligoadenylate synthetase and Mx proteins (36). Instead, HCV clearance correlates closely with intracellular expression of gamma interferon (IFN-γ), thus suggesting that HCV might be more sensitive to IFN-γ than to IFN-α.

IFN-γ is a unique member of the IFN family. Its receptor, signaling pathway, and cellular effects differ from those of IFN-α and IFN-β (2, 3). IFN-γ is secreted by activated T cells and natural killer (NK) cells and may contribute to HCV clearance in several ways. First, IFN-γ enhances NK cell activity (2) and induces the expression of inflammatory and potentially antiviral cytokines such as tumor necrosis factor alpha (27). Second, IFN-γ facilitates induction and effector function of T cells via upregulation of major histocompatibility complex class I and II proteins and promotes antigen processing via induction of immunoproteasomes (9). In this respect, it is noteworthy that frequency and repertoire of HCV-specific, antigen-nonspecifically expanded T-cell lines. None of these immunologic effects were observed in the third chimpanzee injected with an HBV control vector. Despite these immunologic effects of the experimental vector, however, IFN-γ gene transfer did not result in a significant and long-lasting decrease of HCV titers. In conclusion, liver-directed IFN-γ gene delivery resulted in HCV-specific and nonspecific activation of cellular immune responses but did not result in effective control of HCV replication.

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expression to hepatocytes (28). This gene transfer system is based on a recombinant, IFN-γ-expressing HBV vector (rHBV-IFN-γ), which is devoid of all HBV open reading frames and packaged with HBV core (HBc) and surface (HBs) proteins expressed by a second, helper construct. The resulting infectious, recombinant HBV particles should therefore specifically infect hepatocytes via the putative HBV receptor and direct IFN-γ expression via liver-specific HBV promoter and enhancer elements (41). This study represents the first in vivo application of a recombinant HBV vector in nonhuman primates, compares it to a recombinant adeno viral vector (rAd-IFN-γ), and assesses its effects on HCV-specific and nonspecific immune responses and viral titers.

MATERIALS AND METHODS

Chimpanzees. Chimpanzees Ch1535, Ch1536, and CB0561 were housed at New Iberia Research Center, University of Louisiana at Lafayette, under protocols approved by the Animal Care and Use Committees of the New Iberia Research Center, the National Institute for Diabetes and Digestive and Kidney Disease, and the Public Health Service Interagency Model Committee. Ch1535 and Ch1536 had developed persistent HCV infection after intravenous inoculation with HCV-H77 (17). The clinical and virological course of persistent infection has previously been described (20). Serum HCV RNA and alanine aminotransferase (ALT) levels remained relatively constant over the last study periods of 60 and 40 months, respectively, which ended 1.4 years prior to study. The chimpanzees were genotyped at 5.0 log copies/ml for HCV RNA levels and <20 U/liter for ALT levels. Five years and 8 months after the initial HCV infection, Ch1535 and Ch1536 each received an intravenous injection of 1011 enveloped viral particles of rHBV-IFN-γ (week 0) followed by an injection of 2 × 1011 viral particles of rHBV-IFN-γ (week 8) and 1011 infectious units of rAd-IFN-γ (week 15).

CB0561 had developed persistent HCV infection after intravenous inoculation with HCV H77. Ten years and 6 months after its initial HCV infection, CB0561 received an intravenous injection of 2 × 1011 viral particles of rHBV control vector delivering Renilla luciferase (rHBV-Luc; week 0) instead of the IFN-γ gene. One week after injection of the vector, Renilla luciferase mRNA was detectable in the liver at 65.85 RLU/copy/million GAPDH mRNA copies.

Synthetic peptides. Six hundred pentadecamer peptides (Mimotopes, Clayton, Australia), overlapping by 10 amino acids each and spanning the complete HCV genotype 1a (H77) polyprotein sequence (NCBI accession no. AF009606) (17), and the clinical and virological course of persistent infection has previously been described (20). Serum HCV RNA and alanine aminotransferase (ALT) levels remained relatively constant over the last study periods of 60 and 40 months, respectively, which ended 1.4 years prior to study. The chimpanzees were genotyped at 5.0 log copies/ml for HCV RNA levels and <20 U/liter for ALT levels. Five years and 8 months after the initial HCV infection, Ch1535 and Ch1536 each received an intravenous injection of 1011 enveloped viral particles of rHBV-IFN-γ (week 0) followed by an injection of 2 × 1011 viral particles of rHBV-IFN-γ (week 8) and 1011 infectious units of rAd-IFN-γ (week 15).

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specific T-cell responses, individual peptide pools or single peptides (1 μg/ml per peptide) or the corresponding concentration of DMSO was used to stimulate the following for 36 h: (i) 1 × 10^6 peripheral blood CD4^+ T cells or 5 × 10^6 peripheral blood CD8^+ T cells in the presence of 1 × 10^6 irradiated (3,000 rad) autologous PBLs or (ii) 5 × 10^6 intrahepatic CD8^+ T cells, purified from expanded T-cell lines, in the presence of 2.5 × 10^5 of irradiated (3,000 rad) autologous PBLs. To investigate HBV-specific T-cell responses, peptide pools spanning HBc and HBs amino acid sequences were used to stimulate 3 × 10^5 PBLs. All cultures were set up in duplicate in RPMI 1640 containing 5% fetal bovine serum and 2 mM L-glutamine. Cytokine spots were evaluated and counted.

**RESULTS**

**Intrahepatic expression of IFN-γ without increase of serum ALT values.** Two chimpanzees with a well-characterized course of persistent HCV infection, Ch1535 and Ch1536 (17, 20), were intravenously injected with 10^11 viral particles of rHBV-IFN-γ (week 0) and subsequently with 2 × 10^11 viral particles of rHBV-IFN-γ (week 8) and 10^11 infectious units of rAd-IFN-γ (week 15). To assess intrahepatic IFN-γ expression, the relative amount of IFN-γ mRNA was quantitated by use of serial liver biopsy samples. IFN-γ mRNA levels in pretherapy liver biopsy samples were much higher (3.74-fold for Ch1535 and 21.43-fold for Ch1536) than the IFN-γ levels in nine preinfection liver biopsy samples from five other chimpanzees, because preinfection biopsies from Ch1535 and Ch1536 were not available. Gene transfer and intrahepatic expression of IFN-γ were not associated with liver damage in any of the chimpanzees as measured by serum ALT levels (Fig. 1B).

Next, we asked whether the increase in IFN-γ mRNA in the liver biopsy samples represented the transferred human IFN-γ or the endogenous chimpanzee IFN-γ. Because the sequences of the transferred human and endogenous chimpanzee IFN-γ differed in only 3 nucleotides and thus did not allow reliable distinction by quantitative real-time PCR, RT-PCR products were cloned and sequenced. The week 9 biopsy of Ch1536 was chosen for this purpose, because many immunologic and virological parameters showed maximal changes at this time point (see below). Both human and chimpanzee IFN-γ sequence were detectable in the week 9 biopsy of Ch1536, with five of nine molecular clones matching the human IFN-γ sequence and four of nine molecular clones matching the chimpanzee IFN-γ sequence.

In contrast to injection of rHBV-IFN-γ, injection of 10^11 infectious units of rAd-IFN-γ resulted in a transient IFN-γ mRNA peak only in the liver of Ch1536 (Fig. 1A). This apparent lack of gene delivery in Ch1535 could not be attributed to adenovirus-neutralizing antibodies, because both chimpanzees tested negative for adenovirus-neutralizing antibodies prior to and positive after injection of rAd-IFN-γ (not shown). However, as the first liver biopsy was performed not earlier than a week after rAd-IFN-γ injection, a very short and transient period of expression of IFN-γ could not be completely excluded for Ch1535.

**Phenotypic changes of peripheral blood and intrahepatic lymphocyte populations.** To assess whether intrahepatic expression of IFN-γ stimulated the migration of circulating T cells into the liver, we first quantitated intrahepatic CD8β and CD4 mRNA levels by TaqMan PCR, which increased by less than threefold and less than fourfold, respectively, during the study period but not immediately after gene therapy (not
shown). Next, we analyzed the composition of the intrahepatic infiltrate and determined the expression of tissue-homing markers CCR5 and CXCR3 on circulating and intrahepatic T cells by flow cytometry. Whereas no changes in CCR5 expression were detected (not shown), the frequency of circulating CXCR3+ T cells increased after each rHBV-IFN-γ injection and after the rAd-IFN-γ injection (Fig. 2A, upper panels). In agreement with this result, an increased frequency of CXCR3+ T cells in the intrahepatic T-cell population became detectable at the same time points (Fig. 2A, lower panels) and lasted for more than 2 weeks after each IFN-γ gene delivery. These CXCR3+ T cells characteristically displayed a CD3dim phenotype (Fig. 2B). In CB0561, an increased frequency of CXCR3+ T cells was not observed in the circulating lymphocyte population or in the intrahepatic lymphocyte population after injection of rHBV-Luc (Fig. 2A).

Interestingly, IFN-γ gene delivery was also associated with an increase of the percentage of circulating CD16+ CD3+ T cells. One to two weeks after the second injection of rHBV-IFN-γ, for example, CD16+ cells constituted more than 50% of circulating CD3+ T cells in both Ch1535 and Ch1536 (Fig. 2C and D), whereas no increase in the frequency of CD16+ T cells was observed in CB0561 after injection of rHBV-Luc (Fig. 2C). The frequency of T cells that expressed other NK cell markers such as CD56 and/or CD161 also increased but to a lesser extent and later, i.e., more than 4 weeks after each IFN-γ gene delivery (not shown). The expression of CD16, CD56, and CD161 on circulating CD3+ T cells was consistent with similar observations in acute lymphocytic choriomeningitis virus infection in mice (33) and in acute HCV infection in chimpanzees (V. Racanelli and B. Rehermann, unpublished results). In contrast, the percentage of CD3-negative cells with CD16 and/or CD56 expression did not increase significantly.

**IFN-γ production and proliferation of circulating and intrahepatic T cells.** To quantitate HCV-specific T cells, CD8+ and CD4+ T cells were isolated from PBLs and tested separately in IFN-γ ELISPOT assays with 600 overlapping peptides that corresponded to the complete amino acid sequence of the infecting HCV. As shown in Fig. 3, the frequency of circulating, HCV-specific CD8+ (Fig. 3A) and CD4+ (Fig. 3B) T cells increased transiently, especially after the second rHBV-IFN-γ injection, and returned to baseline levels within 2 weeks. Injection of rAd-IFN-γ induced a more delayed increase in the number of IFN-γ-producing, HCV-specific CD8+ (Fig. 3A) and CD4+ (Fig. 3B) cells. In contrast, no increase in the frequency of HCV-specific CD8+ or CD4+ T cells was observed in CB0561 after injection of rHBV-Luc (Fig. 3A and B).

To exclude the possibility that induction of an immune response to the HBV vector itself rather than immune activation by IFN-γ was responsible for these changes, we studied the immune response to overlapping peptides spanning the HBe and HBs sequences. As shown in Fig. 3C, the frequency of HBV-specific T cells did not increase in any chimpanzee.

Because the number of lymphocytes derived from liver biopsy samples was too small to allow direct ex vivo analysis with the complete panel of overlapping HCV peptides, in vitro expansion was required prior to IFN-γ ELISPOT analysis. Intrahepatic lymphocytes were therefore antigen-nonspecifically stimulated with anti-CD3, and cell numbers were compared prior to and after 6 weeks (two cycles of 3 weeks of anti-CD3 stimulation) of in vitro culture. Interestingly, a dramatic increase in the proliferative capacity of biopsy sample-derived T cells was evident after the second and third rounds of IFN-γ gene delivery to Ch1535 and Ch1536 (Fig. 3D). Expanded liver biopsy sample-derived T-cell lines consisted predominantly of CD8+ T cells (>90% [not shown]) and contained HCV-specific, IFN-γ-producing cells (Fig. 3E).

**Fine mapping of CD8+ T-cell responses specific for E1 and NS5B.** To precisely determine the breadth of the HCV-specific T-cell response within representative antigenic regions, T-cell lines derived from the week 9 biopsy samples (1 week after the second rHBV-IFN-γ injection) were tested against single overlapping peptides. As shown in Fig. 4A, 38 single, overlapping peptides corresponding to HCV E1 and 38 peptides corresponding to the amino-terminal third of NS5B were tested individually with T cells from each chimpanzee. Interestingly, only a single epitope region was identified in each protein, and the NS5B epitope was recognized in both chimpanzees (Fig. 4A). Epitope-specific responses were confirmed in IFN-γ secretion assays using the same population of purified CD8+ T cells (Fig. 4B). Collectively, these results suggest that E1 and NS5B-specific T-cell responses remained narrowly focused on a few epitopes despite the apparent overall increase in the vigor of cellular immune responses after IFN-γ gene delivery.

**Lack of significant changes in HCV titer.** Despite modulatory effects on HCV-specific and nonspecific cellular immune responses, however, IFN-γ gene delivery did not result in HCV clearance or in a lasting decrease of serum HCV RNA titer. Whereas Ch1536 demonstrated slight decreases of serum HCV RNA after the second rHBV-IFN-γ injection and after the rAd-IFN-γ injection, these decreases remained within 1 log10 did not last for more than 2 weeks, and were within the range of spontaneous fluctuations in HCV RNA titers at later time points (Fig. 5). Likewise, no significant and lasting decrease in serum HCV RNA titer was observed in Ch1535. Thus, neither direct antiviral effects of liver-directed IFN-γ expression nor the observed activation of HCV-specific and nonspecific cellular immune responses was sufficient for effective HCV control.

**DISCUSSION**

Although IFN-γ was first described almost 40 years ago (42) and has been well characterized at the cellular and molecular levels in in vitro studies, only a few clinical applications have been tested (14). Specifically, its in vivo immunomodulatory effect has not been systematically analyzed in humans or in nonhuman primates. In this study, immunotherapy with rHBV-IFN-γ and Ad-IFN-γ resulted in upregulation of intrahepatic IFN-γ mRNA and the following immunologic observations. First, the frequency of CXCR3+ T cells in the peripheral blood as well as in the intrahepatic lymphocyte population increased significantly. The intrahepatic CXCR3+ T-cell population characteristically displayed a CD3dim phenotype, indicating that it consisted either of recently activated T cells (33) that have downregulated the T-cell receptor or of NK T cells because NKT cells express low levels of CD3 and high levels of CXCR3 on the cell surface (15, 16, 38). Second, each injection of rHBV-IFN-γ resulted in a significant increase in the percentage of circulating CD3+ T cells, which coexpressed NK cell markers such as CD16. The immediate and transient nature of
FIG. 2. Phenotypic changes in peripheral blood and intrahepatic lymphocyte populations. (A) Frequency of CXCR3+ cells in peripheral blood (upper panels) and intrahepatic (lower panels) CD3+ lymphocytes. (B) Representative dot plots demonstrating CXCR3+ expression on intrahepatic CD3+ lymphocytes. (C) Frequency of CD16+ cells in CD3+ peripheral blood lymphocytes. (D) Representative dot plots demonstrating CD16 expression on peripheral blood lymphocytes. The dotted vertical lines in panels A and C indicate the time points of intravenous injection of $10^{11}$ viral particles of rHBV-INF-γ (week 0), $2 \times 10^{11}$ viral particles of rHBV-INF-γ (week 8), and $10^{11}$ infectious units of rAd-INF-γ (week 15) in Ch1535 and Ch1536 and $2 \times 10^{11}$ viral particles of rHBV-Luc (week 0) in CB0561.
this increase suggests transcriptional regulation of CD16 rather than redistribution and/or migration of specific CD3⁺ CD16⁺ T cells. Indeed, upregulation of NK cell markers on CD3⁺ T cells has previously been described in murine models of viral infections and in association with T-cell activation (33). Consistent with these results, an increased frequency of CD16⁺ CD3⁺ T cells was also observed in chimpanzees with acute HCV infection (V. Racanelli and B. Rehermann, unpublished results). Third, an increased frequency of HCV-specific T cells was detected ex vivo in the peripheral blood and in vitro in antigen-nonspecifically expanded, liver biopsy sample-derived T-cell lines. Interesting characteristics of the HCV-specific T-cell response were its narrow focus on a few epitopes within the analyzed E1 and NS5B peptide pools and its transient nature. The narrow focus of the HCV-specific T-cell response may be related to IFN-γ’s capacity to induce immunoproteasomes, which differ from constitutive proteasomes in both qualitative and quantitative aspects of antigen processing. Thus, IFN-γ-induced immunoproteasomes may generate specific T-cell epitopes in larger quantities, as recently demon-

FIG. 3. IFN-γ production and proliferation of circulating and intrahepatic T cells. (A-B) Ex vivo IFN-γ ELISPOT analysis of purified peripheral blood CD8⁺ (A) and CD4⁺ T cells (B) using pools of overlapping HCV peptides spanning the complete amino acid sequence of the infecting HCV. (C) Ex vivo IFN-γ ELISPOT analysis of PBLs using pools of overlapping peptides spanning the entire HBe and HBs amino acid sequence. (D) Antigen-nonspecific proliferation of intrahepatic lymphocytes in response to anti-CD3 and IL-2. The proliferation index reflects the cell number after 6 weeks of culture divided by the cell number at the start of culture. (E) The frequency of HCV-specific, IFN-γ-producing T cells in intrahepatic T-cell lines was determined in IFN-γ ELISPOT assays. CD8⁺ T cells were isolated from antigen-nonspecifically expanded intrahepatic T-cell lines and tested in IFN-γ ELISPOT assays with pools of overlapping peptides spanning the complete amino acid sequence of the infecting HCV. NT, not tested due to poor expansion of intrahepatic lymphocytes. Dotted vertical lines indicate the time points of intravenous injection of 10¹¹ viral particles of rHBV-IFN-γ (week 0), 2 × 10¹¹ viral particles of rHBV-IFN-γ (week 8), and 10¹² infectious units of rAd-IFN-γ (week 15) in Ch1535 and Ch1536 and 2 × 10¹¹ viral particles of rHBV-Luc (week 0) in CB0561. GE, genome equivalent.
strated for an immunodominant HBV core epitope (32). The transient nature of the induced HCV-specific T-cell response (Fig. 3A, B, and E) is interesting in the context of recent reports that IFN-γ/H9253 induces activation-induced cell death of T cells (5, 30). It is therefore tempting to speculate that intrahepatically expressed IFN-γ/H9253 does not only activate HCV-specific T cells but also induces activation-induced cell death. This hypothesis would be in line with the lack of a long-lasting virologic response in the present study.

Whereas the induction of IFN-γ/H9253 mRNA in the liver was clearly shown, it was more difficult to determine whether the immunologic effects were due to human IFN-γ/H9253, transferred by the rHBV-IFN-γ vector, or to subsequently induced endogenous chimpanzee IFN-γ. Both human and chimpanzee IFN-γ mRNAs, which differ by only 3 nucleotides, were detectable in the biopsy sample of Ch1536 taken at week 9, when immunologic parameters showed maximal changes. The following mostly indirect evidence supports the notion that the immunologic effects were caused by human IFN-γ, transferred by the vector, and amplified by chimpanzee IFN-γ, produced by HCV-specific chimpanzee T cells. First, no IFN-γ mRNA was detectable in a control chimpanzee which was injected with rHBV-Luc, thereby confirming that induction of IFN-γ mRNA did not result from a nonspecific response to the vector backbone. Second, only the rHBV-IFN-γ vector and not the rHBV-Luc control vector resulted in an increase in HCV-specific CD4+ and CD8+ T-cell responses, thereby indirectly confirming the role of the vector-expressed human IFN-γ. As shown in Fig. 3, the frequency of HCV-specific IFN-γ-producing chimpanzee CD4+ and CD8+ T cells was increased, thereby amplifying the overall IFN-γ response.

A specific T-cell response to the HBV backbone of the vector was excluded, because there was no increase in the IFN-γ response of HBV-specific T cells in any of the three rHBV-injected chimpanzees (Fig. 3C). This was expected because the vector itself is devoid of all HBV open reading frames, so that there is no in vivo production of HBV antigens in transduced cells. Furthermore, a small amount of antigen, such as the HBV proteins that package the vector, typically does not induce any cellular immune response if injected intravenously and without adjuvants. In summary, we therefore conclude that the observed immunologic changes were initiated by the immune activation by the rHBV-expressed human IFN-γ and subsequently amplified by chimpanzee IFN-γ produced by HCV-specific T cells.

This interpretation applies only to the rHBV-IFN-γ experiments, because the rAd-IFN-γ experiments were not controlled by injection of a control vector into a control animal. Despite the observed modulation of HCV-specific and nonspecific cellular immune responses, IFN-γ gene transfer did not result in a significant and long-lasting decrease of the HCV titer. An effect of species differences between human IFN-γ

FIG. 4. Fine mapping of CD8+ T-cell responses to E1 and NS5B. (A) CD8+ T cells were isolated from T-cell lines established from liver biopsy samples taken at week 9 (1 week after the second rHBV-IFN-γ injection) (Fig. 3D and E) and tested in IFN-γ ELISPOT assays against individual overlapping peptides spanning the E1 sequence and the amino-terminal third of NS5B. (B) IFN-γ secretion assays confirm T-cell epitopes. After 12 h of stimulation with the selected peptide, secreted IFN-γ was captured on the surfaces of lymphocytes of Ch1536 and stained with secondary antibodies. Stimulation with DMSO was performed as a negative control. FSC, forward scatter.

FIG. 5. Serum HCV RNA titers. Serum HCV RNA was quantified by real-time RT-PCR. Dotted vertical lines indicate the time points of intravenous injection of 10^11 viral particles of rHBV-IFN-γ (week 0), 2 × 10^11 viral particles of rHBV-IFN-γ (week 8), and 10^11 infectious units of rAd-IFN-γ (week 15) in Ch1535 and Ch1536 and 2 × 10^11 viral particles of rHBV-Luc (week 0) in CB0561. GE, genome equivalent.
and chimpanzee IFN-γ receptor was excluded as explanation for this virological nonresponse, because human IFN-γ was shown to induce typical effects, such as upregulation of major histocompatibility complex class I/II and immunoproteasome subunits, in chimpanzee fibroblasts in vitro (not shown). Therefore, one alternative explanation is that IFN-γ does not exert any antiviral effects in vivo in the HCV-infected liver. Whereas this contrasts in vitro results of IFN-γ-mediated suppression of subgenomic and genomic HCV RNAs in cell culture (7), it is consistent with the results of a recent clinical trial. In that trial, persistently HCV-infected patients who were treated with recombinant IFN-γ displayed no change in HCV titers despite hematologic changes that indicated effective IFN-γ delivery (34). Notably, peak IFN-γ mRNA levels in the two chronically HCV-infected chimpanzees in our study were not lower than those in liver biopsies of three chimpanzees with self-limited hepatitis C, which we tested prospectively during the acute phase of HCV infection and which displayed a maximum increase in intrahepatic IFN-γ mRNA levels of 5-, 15- and 30-fold over preinfection samples (not shown). Although it is possible that a higher concentration or longer-lasting expression of IFN-γ might be required to be effective in the chronically HCV-infected liver, therapeutic delivery of higher doses of IFN-γ might be difficult to tolerate because of systemic and/or local side effects. Constitutive high-level expression of IFN-γ in the livers of transgenic mice, for example, has been associated with the development of chronic hepatitis (40). Alternatively, the use of recently described constructs with conditional IFN-γ expression (22) may allow restriction of IFN-γ expression to HCV-infected hepatocytes and thereby avoid an overall increase of IFN-γ expression in the liver. Finally, a combination of IFN-γ expressing vectors with vectors that encode HCV antigens and/or cytokines such as IL-12 (26) and IL-23 (12) might be useful for better and longer-lasting expansion of HCV-specific T cells and, ultimately, for effective HCV control.

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